Molecular characterization of canine parvovirus in Iran, 2023

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Abstract

Canine parvo virus 2 causes severe and often fatal gastroenteritis and myocarditis in dogs and puppies. Based on the VP2 gene, this virus is classified into 3 variants: CPV-2a, CPV-2b, and CPV-2c. In present study 35 rectal swab samples were collected from dogs with clinical signs, including vomiting and diarrhea, and cases with positive results from the rapid test kit. Samples were screened with PCR assay to screen for the presence of the virus genome. According to the PCR results, all samples were positive. Out of 35 cases, about 34% received at least one dose of vaccine, and almost 66% were not vaccinated at all. A rapid test was also performed for 34 cases. Rapid test was positive for 91% (31 cases) and negative for 9% (3 cases). Phylogenetic analysis of seven samples, which were submitted for sequencing, revealed that 6 of the present isolates (UT-CPV14 to UT-CPV18 and UT-CPV20) were clustered with CPV-2c isolates and one (UT-CPV19) was clustered with CPV-2b sequences. Homology analysis indicated high similarity

(100%) between isolates (UT-CPV14 to UT-CPV18 and UT-CPV20) and isolates K20172c-1, 12B, IZSSI_2021PA43108idAki, BJ001, and CPV-2c/Sull6/2017. UT-CPV19 showed 100% similarity with isolates 19R113-2, YANJI-2, and 15D184. In the present study, we also analyzed a commercial vaccine phylogenetically. Although the homology results indicated almost 98% similarity between current isolates and vaccine, the vaccine sequence was not clustered with any groups in the phylogenetic tree. These results highlight the importance of constantly monitoring circulating strain antigenic changes and the efficacy of vaccines against them.

Keywords: Canine parvovirus, Dog, Vaccine, Phylogeny , Iran

1. Introduction

Like in other countries, the number of people willing to keep pets, especially dogs and cats, has increased in recent years in Iran (1). Like other mammals, their immune system is responsible for ensuring health, well-being, and longevity and protecting them from outer invaders such as infectious agents (2). Among infectious agents infecting canines, CPV-2, and its variants are considered the most common pathogens distributed universally (3). Despite comprehensive vaccination, canine parvovirus type 2 (CPV-2) remains an ongoing cause of highly contagious, fatal gastroenteritis, particularly in puppies in Iran and the rest of the world (4-7).

The disease is mainly transmitted through fecal-oral route. After infection, it takes 3–7 days incubation period before the onset of clinical signs. Viruses replicate in lymph nodes, and subsequently, many viral particles are released into the bloodstream and then enter the gastrointestinal tract, destroy intestinal cells, and form intranuclear inclusion bodies (8). CPV-2 is a non-enveloped icosahedral virus with approximately 25nm in diameter, containing a linear single-strand DNA (9). The genome includes two major open reading frames that encode two non-structural proteins (NS1 and NS2) and two capsid proteins (VP1 and VP2). The VP2 protein is considered to be responsible for virus antigenic properties and characterizing the virus host range and tissue tropism (10).

It seems that canine parvovirus is derived from panleukopenia virus due to specific mutations at capsid protein VP2, which facilitated the host change and permitted the virus to infect canines and lose the ability to infect feline (11). In 1978, CPV-2 was identified and spread globally between 1978 and 1979. During the 80s, the accumulation of mutations in the original virus (CPV2), which was circulating globally, caused the emergence of two antigenic subtypes named CPV-2a and CPV-2b, and in 2000 and additional antigenic subtypes, CPV-2c was identified (4, 12, 13). According to the previous studies, the genetic difference between the original CPV-2 and the antigenic variants CPV-2a and CPV-2b is determined by five to six aa of VP2 protein, including 87, 101, 297, 300, 305, and 426 residues (4). Furthermore, three subtypes have been shown to differ in residue 426, with types 2a, 2b, and 2c displaying Asn, Asp, and Glu, respectively (14).

Although killed vaccines against CPV-2 are available and can provoke antibody response, prevention of canine parvovirus is mainly achieved through vaccination with modified live vaccines (MLV) which have been known to be able to stimulate both antibody- and cell-mediated immune responses, resulting in strong, long-lasting protection against virulent viruses (7, 15, 16). Despite vaccination, CPV remains one of the major reasons for puppy death (7). The most common reason for this vaccination failure is the interference with maternally derived antibodies, which are transferred to puppies through colostrum, placenta, and milk and can prevent the onset of immunity (16). In addition to inappropriate vaccination schedules regarding the persistence of maternal immunity, vaccination of non-responders, and, even more importantly, circulation of different antigenic variants of the virus (7, 16).

Nowadays, the original type 2 canine parvo virus only exist in commercial vaccines and the other subtypes (CPV-2a, CPV-2b, CPV-2c) are distributed in the world canine population (17). In Iran, Hemmatzadeh and Jamshidi, isolated CPV by utilizing MDCK cell line and electron microscopy for the first time in 2002 (18). The first molecular epidemiology study of CPV was first conducted Firoozjaii et al in 2011, which showed the presence of CPV-2a and CPV-2b subtypes in collected samples (19). According to some previous studies in Iran, CPV-2a and CPV-2b are the predominant subtypes circulating in Iran, and CPV-2c has a lower frequency (4, 6, 19-21).

Since all antigenic subtypes are present and circulating among the dog population in Iran, it is necessary to perform constant monitoring to determine the predominant antigenic type; the aim of

the present study was the phylogenetic analysis of CPV isolated from clinical cases and update previous phylogenetic data reported about CPV from Iran.

2. Material and method

2.1 Sample collection

Thirty-five samples were collected from small animal clinics in Tehran city, Tehran province, north of Iran, between July and October 2023. Fecal swabs were collected from dogs ranging 2-12 months, presented clinical signs including vomiting and diarrhea or cases with positive results of Rapid immunochromatography Antigen test kit (AniGen, Seoul, Korea), and transferred to -20°C. The information on each case (including ages, vaccination situation, and result of rapid test kit) is mentioned in Table 1.

2.2 DNA extraction and PCR

Total DNA was extracted from rectal samples and a commercial live attenuated vaccine (Himmvac® DHPPL vaccine, Korea) using SinaPure One (viral nucleic acid extraction mini kit, Sinaclon Co., Iran) according to the manufacturer's instructions. The polymerase chain reaction (PCR) method using primer pair (CPVF2: AAAAAGAGACAATCTTGCACCA and CPVR2: TGAACATCATCTGGATCTGTACC), was applied to amplify a part of VP2 gene to confirm the presence of CPV-2 by amplification of a 747 bp fragment of the CPV viral genome (22-24). The thermal condition was carried out as follows: initial denaturation step at 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s. A final extension step was performed at 72°C for 10 min. The PCR product was analyzed by electrophoresis on agarose gel (1.5%) stained by ethidium bromide.

2.3 Sequencing and phylogenetic analysis

Among all positive samples, 7 were submitted for sequencing by Codon Genetic Company (Tehran, Iran) using the Sanger sequencing method. Sequences were primarily evaluated with BLAST online tool, and then the quality of sequences was checked by Finch TV software version 1.4.0. After that, sequences were edited and trimmed by using MEGA 7 software. Phylogenetic

analysis was performed by MEGA 7 software using the maximum likelihood method based on the General time reversible model (25). For Phylogenetic analysis, a total of 26 nucleotide sequences from each three genotypes of canine parvovirus (CPV-2a, CPV-2b, CPV-2c) were included in the dataset. The phylogenetic tree reliability was estimated with the bootstrap method of 1000 replicates. Sequences were submitted in GenBank and are available with accession numbers: OQ025284, PP471790, PP471791, PP471792, PP471793, PP471794, PP471795.

3. Results

3.1 PCR results

All 35 samples were shown to be positive in the PCR Test. Out of 35 cases in this study, almost 34% (12 cases) received at least one dose of vaccine, and 66% (23 cases) were not vaccinated at all. CPV rapid detection kit was applied for 34 cases. Rapid test was positive for 91% (31 cases) and negative for 9% (3 cases).

3.2 Phylogenetic analysis

BLAST results revealed that all seven sequences were related to Canine parvovirus. Phylogenetic analysis of sequences indicated that only one isolate (UT-CPV19) belongs to CPV-2b genotype (14.3%), and other sequenced clinical isolates (UT-CPV14 to UT-CPV18 and UT-CPV20) belong to CPV-2c genotype (85.7%) (figure 1). Sequences are available at GeneBank under accession numbers OQ025284, PP471790, PP471791, PP471792, PP471793, PP471794, PP471795. Homology analysis (table 2) revealed that, UT-CPV-14, UT-CPV15, UT-CPV16, UT-CPV17, UT-CPV18, and UT-CPV20 had 100% similarity with isolates K20172c-1 (South Korea, 2017), 12B (Iran, 2021), IZSSI_2021PA43108idAki (Italy, 2021), BJ001 (China, 2019), CPV-2c/Sul6/2017 (Iraq, 2017) and 99.85% similarity with isolate CPV/dog/HCM/20/2013 (Indonesia, 2013). Analysis of the UT-CPV19 showed 100% similarity with isolates 19R113-2 (South Korea, 2019), YANJI-2 (China, 2014), 15D184 (South Korea, 2015), and LONGJING-1 (China, 2015). According to homology results, isolates in the present study (UT-CPV14 to UT-CPV20) showed almost 98% similarity with the vaccine strain used for comparison in the present study.

4. Discussion

The disease caused by canine parvovirus 2, which can cause severe hemorrhagic enteritis in dogs, was primarily recognized in 1978 in the USA and spread among the dog population throughout the world with high morbidity and frequent mortality (26). The nucleotide sequence of the gene encoding for VP2 protein, the main determinator for viral host range and tropism, is used for the classification of canine parvovirus 2 into three genotypes, CPV-2a, CPV-2b, CPV-2c (27-30). In a study by Faraji et al, in 2023, analysis of all positive collected samples, based on VP2 gene, showed that they all belong to the CPV-2a genotype. The phylodynamic results of this study also indicate that this genotype emerged primarily in the central parts of Iran, especially in the Alborz province, and the results of mutational analysis indicate a positive selection pressure of CPV-2a genotype (20). In a study conducted by Nikbakht et al, 50 fecal samples were collected and evaluated for the presence of CPV, by different specific primers, which were selected from different regions of the VP2 gene. According to the results of this study, 18 samples were characterized as CPV-2a genotypes and 32 samples were classified as 2b genotypes (6). In another study by Saei et al, among 35 stool samples collected from healthy and diarrheic dogs. Using specific primers for VP2 gene, ten samples were found to be positive for CPV. Further analysis showed that out of 10 samples, only 1 was classified as CPV-2c genotypes, and the others were categorized as CPV-2a and CPV-2b. the results of this study indicate that CPV-2b genotype is the predominant genotype circulating in the northwest of Iran, while the two other genotypes also affect dogs (21). In Another study done by Ghajari et al, according to the phylogenetic analysis results based on the VP2 gene, CPV-2a was predominant among positive samples (50%), followed by CPV-2c (32.1%) and CPV-2b (17.8%) (4). In another study, Firoozjaii et al by using primers selected from variable regions in VP1/VP2 capsid genes, revealed that out of 44 cases, 39 samples were detected as CPV-2a and 5 others characterized as CPV-2b. This study was the first study that lightened the presence of CPV in Iran (19). In another study by Abedi et al, out of 60 CPV positive samples, 32 (53.3%) were bolnged to CPV-2a and 28 (46.7%) were belonged to CPV-2b (31). According to previous studies, the prevalence of CPV-2b and 2a subtypes seems to be higher than the other one. However, the present study shows a high prevalence of CPV-2c genotype among collected samples (85.7%) and only one sequenced sample was CPV-2b (14.3%). Altogether, these results indicate that all 3 genotypes of CPV are present and circulating in Iran .The phylogenetic analysis also revealed that all CPV-2c detected in this study are clustered with isolates from China, Iraq, South Korea, Iran, Indonesia, and Italy. The CPV-2b isolates in the present study are located

near other isolates from China and South Korea. No CPV-2a were identified in the present study. By comparison of the distribution of CPV-2 genotypes in different years based on previous reports, it seems that, the prevalence of CPV-2b decresed through years but the pravalnce of CPV-2c increased instead (figure 2). This phenomenon may be due to this fact that most of commercial vaccines used for immunization against CPV in puppies contain CPV-2b genotypes.

Vaccination is considered an effective and the main tool in preventing disease; however, despite vaccination, different cases of CPV occur, and reports of vaccination failure are documented (10, 32). One of the major causes of vaccination failure is maternally derived antibody interference, and vaccination age is also a significant risk factor for this phenomenon (32). Common vaccines against CPV are made using the original CPV or CPV-2b variant (16). Wilson et al, showed that a multivalent vaccine containing the CPV-2b variant could induce a cross-reactive serological response against other field strains like CPV-2a and CPV-2c (33). Puppies are routinely immunized against CPV in the first months after birth, beginning in the 6-8 weeks, repeating in 3-4 weeks intervals, finishing around 16 weeks, following an annual vaccination (34). Maternal-derived antibodies against CPV disappear with a linear decrease after birth, and their half-life is about 9-10 days. In most puppies, maternal-derived antibodies are reduced by 8-12 weeks of age to a level that allows vaccination. It has been reported that maternal-derived antibodies will completely diminish by 10-14 weeks of age (35). It is considered that administering the final vaccine dose to puppies less than 16 weeks old when the interference of maternally derived antibodies and the development of immunity is possible, might be one of the major causes of vaccination failure (15). In a study Yip et al, the antigen test was positive for 41.2% of vaccinated and 73.2% of unvaccinated diseases dogs. Molecular assays also were positive for 82.4% of vaccinated dogs and 92.7% of unvaccinated dogs (15). In another study by Singh et al, the molecular results revealed that 75.9% of samples belonged to unvaccinated and 24.1% of samples belonged to vaccinated dogs (36). Based on the results of the present study, 65.7% of positive cases weren’t vaccinated, and 34.3% of cases received at least one dose of vaccine. Among vaccinated dogs in present study, only 3 cases (25%) were fully vaccinated with three doses of vaccine and the others 9 (75%) was only received one or two doses of vaccines. These results remark that, besides other mentioned reasons, incomplete vaccination can also pose the animal at a higher risk for CPV disease.

Age can also be considered a risk factor. Although dogs can get disease at any age, puppies less than 6 months of age are more susceptible (32). In a study performed by Sayed-Ahmed et al, dogs between 0-3 months showed the highest prevalence of CPV (68%) followed by 4-6 months of age (53.3%), and the lowest prevalence was observed in dogs above 6 months of age (20%) (37). In another study by Tagorti, out of 54 CPV-infected cases, 70.37% were between 1-3 months, and 26.63% were above 3 months (38). In a study by Behera et al, the results of the age-wise prevalence study indicated that the infection is higher in age group 3-6 (41.37%) than 1-3 months (27.59%), 6-12 months (27.59%) and above 12 months (3.45%) (39). In the current study, among studied cases, 22 cases were 2 and 3 months (62.86%), 7 cases were 4 and 5 months (20%), 5 cases were 6 and 7 months (14.28%) and only one case was 12 months (2.86%). A comparison of the results of the current study with those of the previously mentioned studies can indicate that puppies younger than 6 months are more susceptible to CPV disease than those above 6 months.

Although clinical presentations are valuable for diagnosing CPV, this kind of diagnosis is not definitive since different pathogens can cause diarrhea in dogs, and detection must always be confirmed via a laboratory test. Immunochromatographic-based rapid test kits are advantageous because of their lower price and ease of use. However, the efficacies of these rapid test kits are often dubious (40). In a study by Tinky et al., a PCR test showed 44% of the samples evaluated were positive, while an immunochromatographic strip test showed 36% of samples were positive (40). In another study performed by Mohyedini et al, the ability of immunochromatographic (IC) test to detect CPV infection in 50 PCR-positive samples was evaluated. Out of 50 samples, the IC test detect CPV in 42 samples (84%) (41). In the present study, the PCR test showed that 100% of cases were positive for CPV, while the result of the rapid test kit presented that 91% of cases were positive for CPV and showed 9% of cases as negative. This result indicates the importance of molecular tests alongside rapid test kits when clinical cases represent CPV signs but the rapid tests are negative.

In Iran different commercial vaccinaes are used for immunization of puppies against CPV. These vaccines are live attenuated multivalant vaccines which can be used for immunization against other disease such as Distemper, Hepatitis, Parainfluenza, Leptospira, Laryngoteracheaitis and Tracheobronchitis. Some of these vaccines are HIPRADOG 7®, produced by HIPRA company and contain canine parvovirus 2c genotype strain C-780916, Biocan® Novel DHPPI which

produced by Bioveta company and contains the CPV-2b strain of canine parvovirus, Nobivac® DHPPi produced by MSD company and contain strain C154 of canine parvovirus, CANVAC® produced by DYNTEC company and contain T-86 strain of canine parvovirus and Himmvac® produced by KBNP company, and used in present study. In the present study, we performed a phylogenetic analysis of CPV isolated from clinical cases. Results revealed that 6 samples out of 7 were considered as genotype CPV-2c while the other isolates were characterized as CPV-2b, and no CPV-2a were isolated. Constant monitoring of canine parvovirus and assessment of the efficacy of available vaccines is strongly recommended for finding probable mutations that may affect vaccine-induced immunity. In addition, whole genome sequencing of circulating parvovirus will be helpful in this propose.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contributions

Study concept and design: Arash Ghalyanchilangeroudi

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Drafting of the manuscript: Shabnam Babazadeh, Soroush Sarmadi, Omid Eghbali, Arian Abbassioun, Alireza Bakhshi, Fahimeh Jamiri

Ethic

We declare that all ethical standards related to animal health and welfare have been respected in present study.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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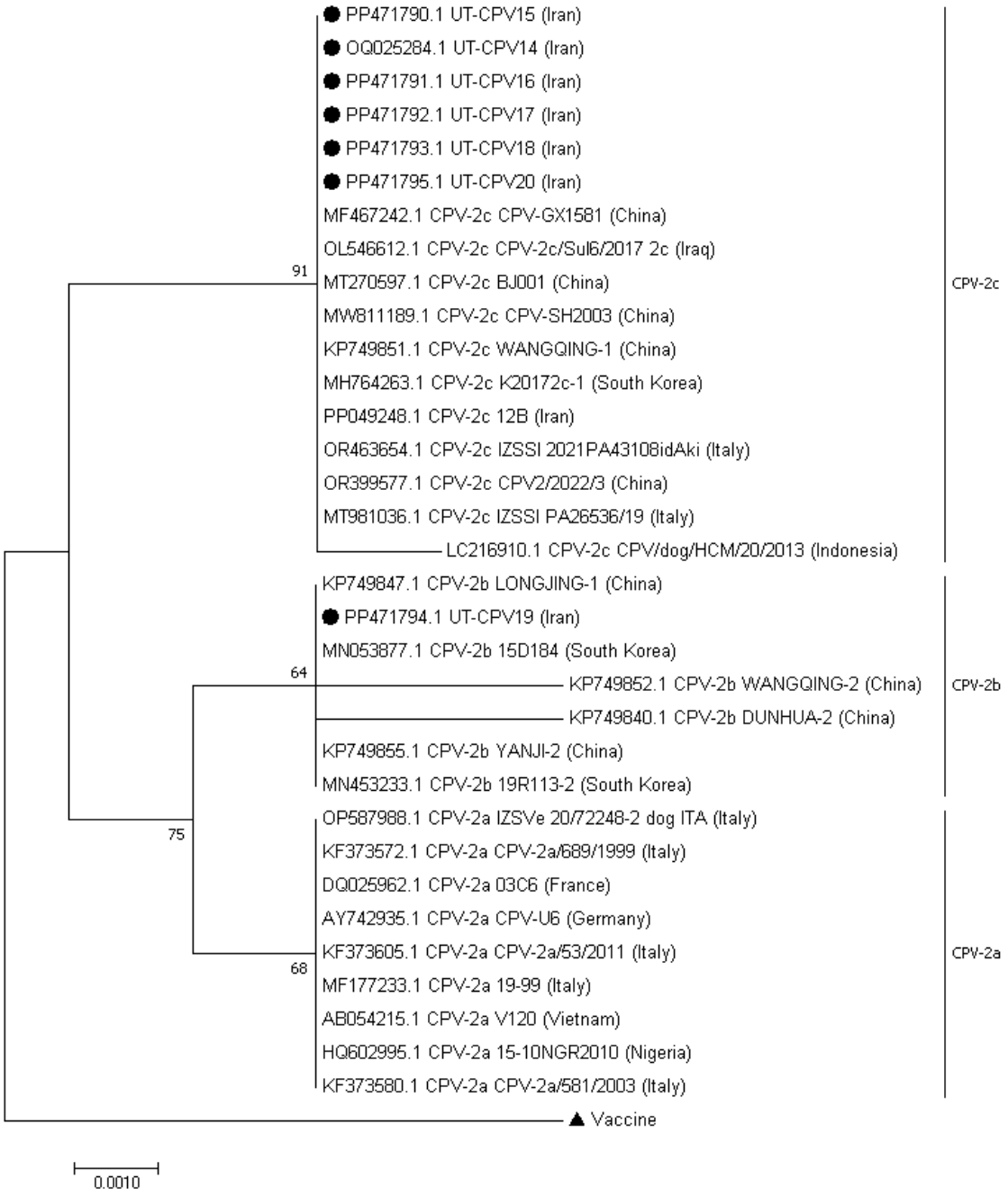
Case number	Age (month)	Vaccination situation	Rapid test results
1	2	No vaccine	Positive
2	2	No vaccine	Positive
3	2	No vaccine	Positive
4	2	No vaccine	Positive
5	2	No vaccine	Positive
6	2	No vaccine	Positive
7	2	No vaccine	Positive
8	2	No vaccine	Not applied
9	3	1 dose	positive
10	3	1 dose	positive
11	3	No vaccine	positive
12	3	No vaccine	Positive
13	3	No vaccine	Positive
14	3	No vaccine	Positive
15	3	1 dose	Positive
16	3	1 dose	Positive
17	3	No vaccine	Positive
18	3	No vaccine	Positive
19	3	No vaccine	Negative
20	3	3 doses	Positive
21	3	No vaccine	Positive
22	3	No vaccine	Positive
23	4	No vaccine	Negative
24	4	No vaccine	Positive
25	4	1 dose	Positive
26	4	1 dose	Positive
27	4	3 doses	Positive
28	5	1 dose	Positive
29	5	No vaccine	Positive
30	6	1 dose	Negative
31	6	No vaccine	Positive
32	6	2 doses	Positive
33	6	3 doses	Positive
34	7	No vaccine	Positive
35	12	No vaccine	Positive

Table 1. Information on cases involved in the present study



٣٦٢ Figure 1- Molecular genetic analysis based on VP2 gene by using the maximum liklihood method and
٣٦٣ General time-reversible model. The tree was generated by comparison of the present sequence with 26 other
٣٦٤ sequences retrieved from NCBI GeneBank. According to the tree out of 7 sequenced samples, 6 were
٣٦٥ clustered with CPV-2c types, and 1 was clustered with CPV-2b types. Isolates in the present study are
٣٦٦ marked with black circles, and the vaccine used in the current study is marked with black triangles.

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		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	UT-CPV14 (OQ025284.1)																			
2	UT-CPV15 (PP471790.1)	100																		
3	UT-CPV16 (PP471791.1)	100	100																	
4	UT-CPV17 (PP471792.1)	100	100	100																
5	UT-CPV18 (PP471793.1)	100	100	100	100															
6	UT-CPV20 (PP471795.1)	100	100	100	100	100														
7	K20172c-1 (MH764263.1)	100	100	100	100	100	100													
8	12B (PP049248.1)	100	100	100	100	100	100	100												
9	IZSSI_2021PA43108idAki (OR463654.1)	100	100	100	100	100	100	100	100											
10	BJ001 (MT270597.1)	100	100	100	100	100	100	100	100	100										
11	CPV-2c/Sul6/2017 (OL546612.1)	100	100	100	100	100	100	100	100	100	100									
12	CPV/dog/HCM/20/2013 (LC216910.1)	99.85	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9								
13	UT-CPV19 (PP471794.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3							
14	19R113-2 (MN453233.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	100						
15	YANJI-2 (KP749855.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	100	100					
16	15D184_(MN053877.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	100	100	100				
17	LONGJING-1 (KP749847.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	100	100	100	100			
18	19-99 (MF177233.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	99.7	99.7	99.7	99.7	99.7		
19	CPV-2a/581/2003(KF373580.1)	99.41	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.4	99.3	99.7	99.7	99.7	99.7	99.7	100	
20	Vaccine	98.95	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.4	98.2	98.4	98.4	98.4	98.4	98.4	98.4	98.4

٣٦٨ .

٣٦٩ Table 2- Nucleotide sequence variation for canine parvovirus virus VP2 segment of clinical samples and vaccine in the present study compared

٣٧٠ with previous sequences retrieved from NCBI GeneBanck

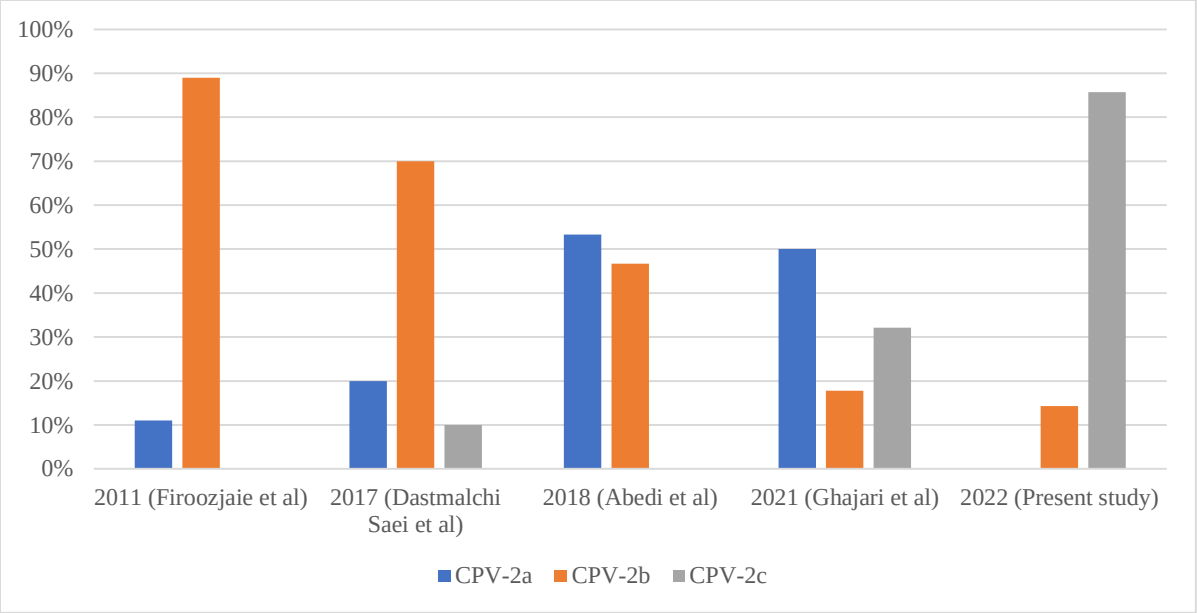

Figure 2- Distribution of CPV-2 genotypes in Iran in different years, based on previouse studies.